

## Molecular Changes in Maspin and Box Genes in Nitrosamine Induced Hepatocarcinogenesis

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**Abstract:** This study was designed to evaluate the potential protective antioxidant and antihepatocarcinogenic effects of beetroots in rats administered nitrosamine precursors (dibutylamine (DBA) and sodium nitrate). Also to monitor the changes in maspin and box genes in parallel with the progression of nitrosamine induced oxidative stress. Rats were divided into four groups (n = 25 each). Group I was treated with DBA/NaNO<sub>3</sub>, group II was treated with DBA/NaNO<sub>3</sub>+ beetroots, in group III rats were treated with beetroots alone and group IV was control group. Markers of oxidative stress and the hepatocarcinogenesis marker Alpha-Feto Protein (AFP) were estimated in serum after 3, 6, 9 and 12 months. Simultaneously, the DNA levels of both cancer related genes maspin and Box were evaluated in liver. The data demonstrated that beetroots was able to antagonize the DBA/NaNO<sub>3</sub>-induced oxidative stress, where the levels of nitric oxide (NO), lipid peroxidation (LPO) and reduced glutathion (GSH) were improved in beetroots protected rats compared to the DBA/NaNO<sub>3</sub>-treated rats. This was accompanied with reduction in the level of AFP. The DNA content of maspin gene in precarcinogenated animals decreased in time dependant manner, whereas the level of box DNA content was increased in parallel with the oxidative stress progression. No significant changes were observed in DNA content of both genes in beetroots-treated group and control group. Histologically, nitrosamine precursors showed distinct alterations such as loss of lobular architecture, fibrosis and the nuclei of many hepatocytes appeared malignant. The antioxidative/antihepatocarcinogenesis effect of beetroots was reflected on the histopathological architecture of liver and hepatocytes.

**Key words:** Box, beetroots, hepatocarcinogenesis, maspin, oxidative stress

### INTRODUCTION

Nitrosamine precursors (Dibutyl amine and sodium nitrite) are categorized as potent hepatocarcinogen in experimental animals. Nitrosamines are predominant compounds in human environment, found in food (such as crude meat and fish) and tobacco (Preussmann *et al.*, 1984; Xia *et al.*, 2005) in addition to the occupational exposure (Straif *et al.*, 2000). Medicinal plants such as beetroots (*Beta vulgaris*) could be used to protect normal (Váli *et al.*, 2007) and diabetic (Ozsoy-Sacan *et al.*, 2007) livers. Also, beetroots showed a protective effect against carbon tetrachloride- and N-nitrosodiethylamine-induced oxidative stress in rats (Kujawska *et al.*, 2009). Oxidative stress has long been known to be involved in the pathophysiology of many human diseases including, but not restricted, to cancer. The term "Oxidative stress" refers to a cell's state characterized by excessive production of Reactive Oxygen Species (ROS) and/or a reduction in antioxidant

defenses responsible for their metabolism. This generates an imbalance between ROS production and removal in favor of the former (Franco *et al.*, 2008). Nitric oxide (NO) is an extremely unstable molecule, it is rapidly converted to nitric and nitrate *in vivo* and *in vitro*, therefore serum nitrite and nitrate levels have been used as an index for NO production (Hunt and Golian, 1992). Cancer is associated mainly with chronic inflammations. This suggests that prolonged cell damage by chronic inflammation is critical in carcinoma development. Over production of NO and its derivatives (peroxynitrite) has been implicated in tissues damage caused by inflammation, contributing the tumor promotion (Nishikawa *et al.*, 1998). Lipid peroxidation, plays an important role in carcinogenesis (Banakar *et al.*, 2004), and it is the most studied biologically free radical chain reaction, where it may lead to the formation of several toxic byproducts such as malondialdehyde (MDE) and 4-hydroxynonenal, which can attack cellular targets including DNA, inducing mutagenicity. Many reports

indicated that glutathione (GSH) and enzymes cooperating with it play a crucial role in cell defense against reactive oxygen species (Hayes and McClellan, 1999; Estrela *et al.*, 2006).

Maspin is a member of the serpin family of serine proteases and functions as a tumor suppressor gene and effective inhibitor of angiogenesis (Zhang *et al.*, 1999). Maspin sensitizes to induce apoptosis (Jiang *et al.*, 2002). Some of the Bcl-2 family members are proapoptotic (Bax) and function as promoters of apoptosis, whereas others are antiapoptotic (bcl-2-bcl-x1) and work as repressors (Adams and Cory, 1998).

This study aims to find out the role of beetroots as a natural protective agent against the carcinogenicity of nitrosamine precursors and to study of the maspin and bax genes as molecular markers and the changes in NO, LPO, GSH and AFP as biomarkers in hepatocarcinogenesis.

## MATERIALS AND METHODS

**Chemical and reagents:** Both Dibutyle amine (BDH limited Pool, England) and sodium nitrite (Sigma Diagnostic Inc., USA) were used to induce the hepatocarcinogenesis in rats. PCR reagents (10X buffer, *taq* DNA polymerase, dNTPs and the DNA marker  $\Phi$ x174) were purchased from Promaga (Promega, USA), whereas primers were purchased from Sigma (Sigma Diagnostic Inc, USA). Beetroots were administrated with the diet in a concentration of 100 gm/day. Phenol chloroform reagent (Sigma) was used for DNA extraction following the manufacturer instruction.

**Animals and grouping:** This work was carried out in the National Cancer Institute, Cairo, during the period 2008-2009. The study used 100 male Albino rats, weighting 40-50 g, purchased from the animal house colony of the National Cancer Institute (NCI), Cairo University, Egypt. Animals were housed under normal environmental conditions of standard temperature, humidity and diurnal environment of light and dark and fed a standard diet, which composed of (24% proteins, 5.55% fibers, 5.5% ash) and drink tap water *ad libitum*. Animals were divided into four groups, 25 rats each.

Group 1: included rats, which were orally administrated with nitrosamine precursors, Dibutyl amine (1000 ppm) and sodium nitrite (2000 ppm) in water for 12 months (Saad *et al.*, 2007).

Group 2: included rats, which were orally administrated with nitrosamine precursors in water and beetroots for 12 months. Group 3: included rats, which were fed beetroots in diet and normal tap water.

Group 4: Included rats, which were fed standard diet and drink tap water.

**Sampling:** Rats in different groups were, serially scarified, where 6 rats from each group were killed every 3 months, the whole liver was stored at -80°C until used. A portion of liver was fixed in 10% formalin, sectioned in paraffin, stained with haematoxyline and eosin using the standard protocol and histologically examined by a pathologist. For biochemical investigations, serum was separated by centrifugation and stored at -70°C until analyzed.

**DNA extraction:** DNA was extracted from liver tissues, where about 100-150 mg of tissue was placed in 500 mL of digestion buffer (10 mM Tris-HCl, pH 8.0, 10 mM NaCl, 2 mM EDTA, 0.1 mg.mL<sup>-1</sup> DTT, 1% SDS, 1 mg/mL proteinase K) and incubated at 65 °C with gentle agitation for 2-3 hrs. The digest was extracted twice with an equal volume of phenol/chloroform and incubated for 5 min at room temperature. DNA was precipitated with 1/10 volumes of 3M sodium acetate (pH 5.3) and 2 volumes of 100% ethanol. DNA was recovered by centrifugation, washed with 70% ethanol, air-dried, and resuspended in 10 mM TE buffer. DNA preparations were incubated with 1 mL of DNase-free RNase (Boehringer Mannheim) for 1 hr at 37°C, and DNA was re-extracted with phenol/chloroform.

### Biochemical investigations:

**Determination of nitric oxide:** The determination of nitric oxide level in serum was performed using Biodiagnostic Nitrite Assay kit, according to the method described by Berkels *et al.*, (2004). In acid medium and in the presence of nitrite the formed nitrous acid diazotise sulphanilamide and the product is coupled with N-(1-naphthyl)-ethylenediamine. The resulting azo dye has a bright reddish-purple color, which is measured at 540 nm.

**Determination of Lipid peroxide ((Malondialdehyde) in serum:** Total malondialdehyde (MDA) was estimated in according to the method of Ohakawa *et al.* (1979). According to this method Thiobarbituric acid (TBA) reacts with MDA in acidic medium at temperature of 95°C for 30 min to form thiobarbituric acid reactive product the absorbance of the resultant pink product is measured at 534 nm.

**Determination of reduced glutathione in serum:** Reduced glutathione (GSH) was measured according to the method developed by Beutler *et al.* (1963). The method based on the reduction of 5, 5' dithiobis (2 - nitro benzoic acid) (DTNB') with GSH to produce a yellow compound. The reduced chromogen is directly proportional to GSH concentration and its absorbance is measured at 405 nm.

**Determination of AFP in serum:** AFP was determined by microparticle Enzyme Immunoassay (MEIA)

Table 1: Primers used in amplification of Bax and Maspin genes

Gene	Type	Location	Sequence
Bax	Foreword	35-55	5'-GGCCCAACAGCTCTGAGCAGA-3'
	Reverse	491-511	5'GCCACGTGGGCGTCCCAAAGT-3
Maspin	Foreword		5'-GAA GCT GCT GAT GGT CCT CAT GCT GGC-3'
	Reverse		5'-TCTGGC CAT AGT CTG TAG CCC TCT GAG C-3'

according to the method described by Abbot Laboratories diagnostic division (Chicago, USA) using IMX automated bench top immunohistochemistry analyzer system.

### Molecular Investigations:

**Amplification and quantization of maspin and Bax genes:** To detect bax and maspin genes fragments by PCR 1 mM of DNA was amplified in 50  $\mu$ l PCR mixture containing: 1X of magnesium-free PCR 10X buffer (100 mmol/L Tris-HCL (PH 8.8), 500 mmol/L KCL, 15 mmol/L  $MgCl_2$ , 0.1(w/v) gelatin), 200 mM each dATP, dCTP, dGTP and dTTP, 1 mM of each Bax or Maspin primer pairs (Table 1), 1.5 mM (3  $\mu$ l)  $MgCl_2$ , and 2.5 U (0.25  $\mu$ l) *Taq* DNA polymerase. The reaction mixture was completed to 50  $\mu$ l with ddH<sub>2</sub>O. Also, DNA free reaction was performed in parallel as a negative control. Reactions were subjected to a thermalcycling program. For Bax amplification the program consisted of a single initial denaturation step at 94°C for 10 min followed by 38 cycles. Each cycle consisted of denaturation at 94°C for 1 min, annealing at 60°C for one min, and primer extension step at 72°C for 30 sec. These cycles were followed with a single extension step at 72°C for 5 minutes For maspin amplification the cycling program consisted of 38 cycles each consists of at 72°C denaturation at 94°C for 1 min, annealing at 60°C for 1 min and primer extension for 30 sec, followed by a single extension step at 72°C for five minutes. At the end of the reactions, the amplification products were electrophoresed in 2% agarose gel containing ethidium bromide, visualized and photographed under UV transilluminator. The DNA of both Maspin and Bax were quantitated by ethidium bromide staining and gel densitometry.

**Histopathological examination:** The liver tissues for histopathological studies were placed in 10 % formalin, then processed in hematoxyline and eosin stain according using the standard staining method.

**Statistical analysis:** The obtained data were presented as (mean $\pm$ standard error). The difference between two groups were calculated using independent student *t*-test, while the differences between more than two groups were calculated using F-test. Duncan's multiple range tests was used to compare between means of at probability 5%. Statistical analysis was performed using Graphpad-Instat software package (Graphpad, San Diego, CA, USA).

## RESULTS

**Biochemical changes in oxidative stress markers:** The data demonstrated that the mean levels of the NO in the

precarcinogen-treated group were (35.08 $\pm$ 3.69, 42.54 $\pm$ 2.73, 67.81 $\pm$ 1.93, 77.75 $\pm$ 4.38), which showed a significant increase when compared to the time matched mean levels of the control group (7.50 $\pm$ 0.44, 10.01 $\pm$ 1.31, 11.60 $\pm$ 0.85, 12.70 $\pm$ 0.93) at 3,6,9,12 month, respectively ( $p < 0.01$ ). The mean levels of the NO of the precarcinogen in combination with beetroots-treated group were (16.82 $\pm$ 0.87, 21.25 $\pm$ 1.05, 24.02 $\pm$ 1.60, 27.84 $\pm$ 1.70) at 3, 6, 9, 12 month of treatment periods, respectively, ( $p < 0.01$ ), which showed an increase when compared to the mean levels of nitric oxide of the control group at 3, 6, 9, 12 month of the treatment periods respectively. However, the mean levels of NO in the beetroots-treated group were (8.67 $\pm$ 0.70, 10.90 $\pm$ 39.66, 12.07 $\pm$ 0.72, 13.57 $\pm$ 2.14) at 3,6,9,12 month respectively, which did not showed statistically changes when compared to the mean levels of NO of the control group, (Table 2).

The mean levels of the lipid peroxidation of the precarcinogen-treated group were (3.90 $\pm$ 0.2, 4.78 $\pm$ 33, 6.83 $\pm$ 0.47, 8.32 $\pm$ 0.35), which showed a significant increase compared to the mean levels of the control group (1.78 $\pm$ 0.19, 2.13 $\pm$ 0.19, 2.45 $\pm$ 0.17, 2.91 $\pm$ 0.34) at 3,6,9,12 month of the treatment periods, respectively, ( $p < 0.01$ ), whereas the mean levels of the LPO of the precarcinogen combined with beet roots LPO of the precarcinogen in combination with beet roots-treated group was (3.07 $\pm$ 0.31, 3.90 $\pm$ 0.50, 5.05 $\pm$ 0.30, 6.38 $\pm$ 0.55) at 3.6.9.12 month of treatment periods respectively ( $p < 0.01$ ), which showed a significant increase when compared to the mean levels of the LPO of the control group. Non significant variations in the serum LPO mean levels among beet roots-treated group (2.08 $\pm$ 0.22, 2.50 $\pm$ 0.45, 2.74 $\pm$ 0.28, 3.32 $\pm$ 0.30) at 3, 6, 9, 12 months respectively, when compared to the control group (Table 2).

The mean levels of the reduced glutathione of the precarcinogen-treated group were (16.51 $\pm$ 0.94, 14.89 $\pm$ 0.73, 13.10 $\pm$ 0.70, 11.29 $\pm$ 1.09) which showed a significant decrease when compared to the mean levels of GSH of the control group (27.86 $\pm$ 0.54, 28.90 $\pm$ 0.59, 30.80 $\pm$ 0.65, 26.67 $\pm$ 0.86) at 3, 6, 9, 12 month of the treatment periods respectively, ( $p < 0.01$ ), whereas the mean levels of the GSH of the precarcinogen in combination with beet roots-treated group were (19.81 $\pm$ 0.97, 21.76 $\pm$ 0.74, 23.73 $\pm$ 0.73, 25.25 $\pm$ 1.08) at 3, 6, 9, 12 month of the treatment periods respectively, ( $p < 0.01$ ), which showed a decrease when compared to the mean levels of GSH of the control group. In beet roots treated group, the mean levels of GSH were (29.10 $\pm$ 0.66, 30.90 $\pm$ 0.67, 31.70 $\pm$ 0.58, 27.74 $\pm$ 0.69) at 3, 6, 9, 12 months of the treatment periods, respectively

Table-2: Effect of nitrosamine precursors and beet roots administration on the nitric oxide, serum LPO, serum GSH and AFP mean level (mmol.L<sup>-1</sup>)

		Period of treatment (Months)			
Group		3	6	9	12
Nitric Oxide (µmol/L)	Control	7.50±0.44	10.01±1.31	11.60±0.85	12.70±0.93
	Carcinogen	35.08±3.69 <sup>a*</sup>	42.54±2.73 <sup>a</sup>	67.81±1.93 <sup>***</sup>	77.75±4.38 <sup>***</sup>
	Carcinogen+ beetroots	16.82±0.87 <sup>***, b+</sup>	21.25±1.05 <sup>a*, b+</sup>	24.02±1.60 <sup>***, b+</sup>	27.84± 1.70 <sup>***, b++</sup>
	beet roots	8.67±0.70 <sup>a*</sup>	10.90±39.66 <sup>a*</sup>	12.07±0.72	13.57±2.14 <sup>a*</sup>
Serum LOP (µmol/L)	Control	1.78±0.19	2.13±.19	2.45±.17	2.91±0.34
	Carcinogen	3.90±0.20 <sup>**</sup>	4.78±0.33 <sup>**</sup>	6.83±0.47 <sup>***</sup>	8.32±0.35 <sup>***</sup>
	Carcinogen+beetroots	3.07±0.31 <sup>***, b++</sup>	3.90±.50 <sup>***, b+</sup>	5.05±.30 <sup>***, b++</sup>	6.38±0.55 <sup>***, b++</sup>
	beet roots	2.08±0.22 <sup>a*, b**</sup>	2.50±.45 <sup>a, b**</sup>	2.74±.28 <sup>a, b**</sup>	3.32±0.30 <sup>a, b**</sup>
Serum GSH (µmol/L)	Control	27.86±0.54	28.90±0.59	30.80±.65	26.67±0.86
	Carcinogen	16.51±0.94 <sup>***</sup>	14.89±0.73 <sup>***</sup>	13.10±0.70 <sup>***</sup>	11.29±1.09 <sup>***</sup>
	Carcinogenbbeet roots	19.81±0.97 <sup>***, b++</sup>	21.76±0.74 <sup>***b**</sup>	23.73 ±.73 <sup>***b**</sup>	25.25±1.08 <sup>***b**</sup>
	beet roots	29.10±0.66 <sup>a*</sup>	30.90±0.67 <sup>***</sup>	31.70± .58 <sup>a*</sup>	27.74±0.69 <sup>a*</sup>
Serum AFP (µmol/L)	Control	2.04 ± 0.32	2.80±.28	2.42±.32	2.60±.55
	Carcinogen	1.50 ± .45 <sup>a*</sup>	2.72±.45 <sup>a*</sup>	7.31±52 <sup>***</sup>	17.60±1.33 <sup>***</sup>
	Carcinogenbbeet roots	0.80 ± 0.06 <sup>***, b*</sup>	1.60±.93 <sup>a*, b*</sup>	5.34±.93 <sup>***, b*</sup>	8.45±1.11 <sup>***, b**</sup>
	beet roots	1.52±0.43 <sup>a*, b</sup>	1.73±0.63 <sup>a*, b+</sup>	2.90±.52 <sup>b++</sup>	3.65±0.55 <sup>a*, b++</sup>

Data is represented as mean±standard error, (a) and (b) refer to statistical differences between the corresponding group versus the control or the carcinogen group, respectively. (+) and (++) refer to significant changes at p<0.05, p<0.01

which did not showed statistically changes when compared to the mean levels of GSH of the control group (Table 2).

The mean levels of the AFP of the precarcinogen-treated group were (1.50±0.45, 2.72±0.45, 7.31±0.52, 17.60±1.33) which showed a significant increase when compared to the mean levels of AFP of the control group (1.04±0.32, 1.80± 0.28, 1.42±0.32, 2.60±0.55) at 3,6,9,12 month of the treatment periods respectively (p<0.01), whereas the mean levels of the AFP of the precarcinogen in combination with beetroots-treated group were (1.80±0.06, 2.60±0.93, 5.34±0.93, 8.45±1.11) at 3.6.9.12 month of treatment periods respectively, which showed an increase when compared to the mean levels of the AFP of the control group. Non-significant variation in the AFP mean levels among beet roots-treated group (1.52±0.43, 1.73±0.63, 2.20±0.52, 3.15±0.55) at 3, 6,9,12 month respectively, when compared to the control group (Table 2).

**Molecular changes in both maspin and bax genes:** The DNA product of maspin gene was negatively affected with the induction of hepatocarcinogenesis, where the gene-banding pattern has decrease in time dependant manner compared to the control (Fig. 1a). By the end of DBA/NaNO<sub>3</sub>-treatment period (12 moths) the gene product decreased by 30% compared to the normal rats (Fig. 1c). A similar banding pattern was obtained in DBA/NaNO<sub>3</sub>-beetroots-treated group (Fig. 1b). However, in beetroots-treated group no significant changes were obtained.

An opposite banding pattern was observed in the amplification of bax. In DBA/NaNO<sub>3</sub>-treated rats the DNA content have gradually increased (Fig. 2a). By the end of treatment period (12 months), the bax DNA was amplified 64% more than the control level (Fig. 2c).

Similarly rats dually treated with the precarcinogen and beetroots showed a similar banding pattern (Fig. 2 b). Non significant change in DNA band in the beetroots-treated group and control group.

**Histopathological investigations:** Photomicrograph of control liver section of rats is represented in Fig. 3 showed normal architecture. Hepatocytes were polyhedral in shape; their cytoplasm was granulated with small uniform nuclei. Hepatocytes were arranged in well-organized cords, the cords were separated by narrow blood sinusoids. Photomicrograph of liver section of rats administrated with beet roots alone exhibited normal architecture during the different periods of treatment (3, 6, 9, 12 month), this indicating the non-toxic nature of beet roots. Examination of liver section of rats treated with nitrosamine precursors showed distinct alterations after 9 months as compared to untreated control group, such as loss of lobular architecture, fibrosis and fatty infiltration, also the nuclei of many hepatocytes appeared malignant. Although, administration of rats with beet roots with nitrosamine precursors, hemotoxyln and eosin section of liver of rats showed none variation in liver section as compared to nitrosamine precursors-treated rats after 9 months of treatment (Fig. 3).

## DISCUSSION

This work focused on the changes in the pattern of the oxidative stress markers, AFP and the cancer related genes maspin and bax in the liver of rats received beetroots during hepatocarcinogenesis induced by nitrosamine precursors namely dibutylamine and sodium nitrite. Nitrosamine precursors mediate their mutagenic effects by causing DNA damage, oxidative stress, lipid peroxidation which lead to increased cellular degeneration

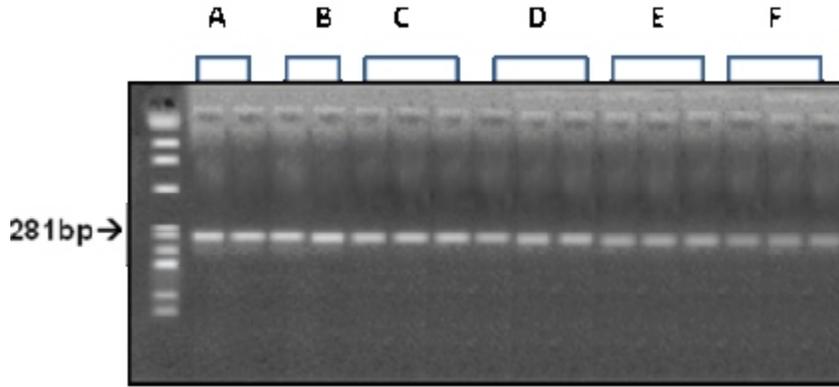


Fig. 1a: Agarose gel electrophoresis of PCR amplified maspin gene (281 bp fragment) in carcinogenated rats. M: DNA marker, Lanes A were loaded with DNA of control group after 3 and 12 months, respectively. Lanes B were loaded with DNA of beetroots-treated rats after 3 and 12 months, respectively. Lanes C, D, E and F were loaded with DNA of animals exposed to the carcinogen precursors for 3, 6, 9 and 12 months, respectively

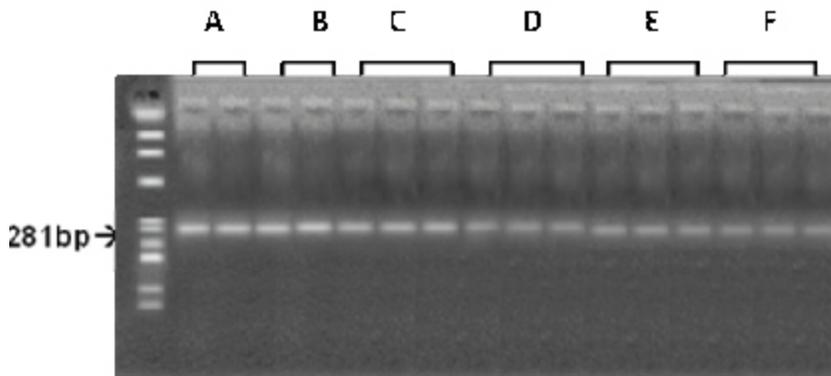


Fig. 1b: Agarose gel electrophoresis of PCR amplified maspin DNA (281 base pair) after oral administration of the (carcinogen + beetroots). M: DNA marker, Lane A was loaded with DNA of control group after 6 and 9 months, respectively. Lanes B were loaded with DNA of beetroots-treated group after 6 and 9 months, respectively. Lanes C, D, E and F were loaded with DNA of (carcinogen + beetroots)-treated group after 3, 6, 9 and 12 months, respectively

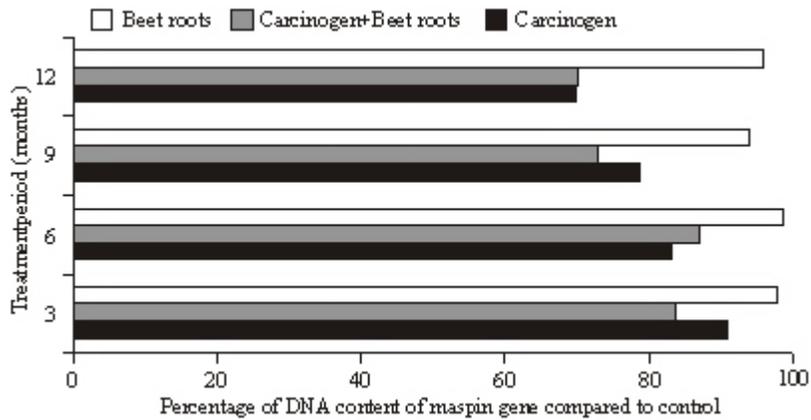


Fig. 1c: Percent of DNA content of maspin gene in carcinogen, carcinogen+beetroots-treated and beetroots-treated animals compared to the control levels

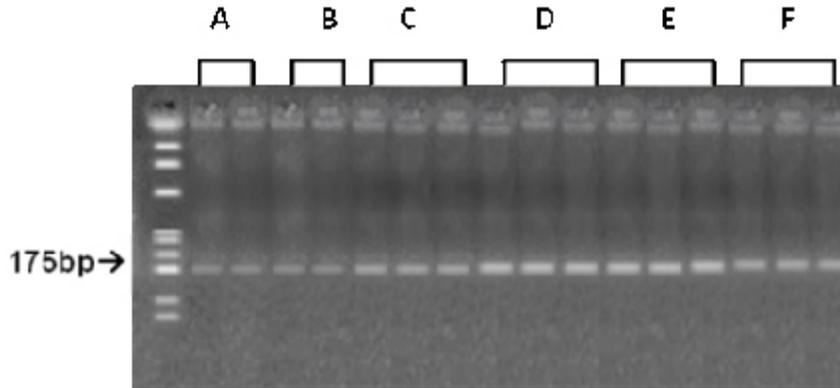


Fig. 2a: Agarose gel electrophoresis of PCR amplified Bax gene (175bp fragment) in precarcinogenated rats. M: DNA marker, Lanes A were loaded with DNA of the control group after 3 and 12 months, respectively. Lanes B were loaded with DNA of rats treated with beetroots for 3 and 12 months, respectively. Lanes C were loaded with DNA of rats treated with the carcinogen for 3, 6, 9 and 12 months, respectively

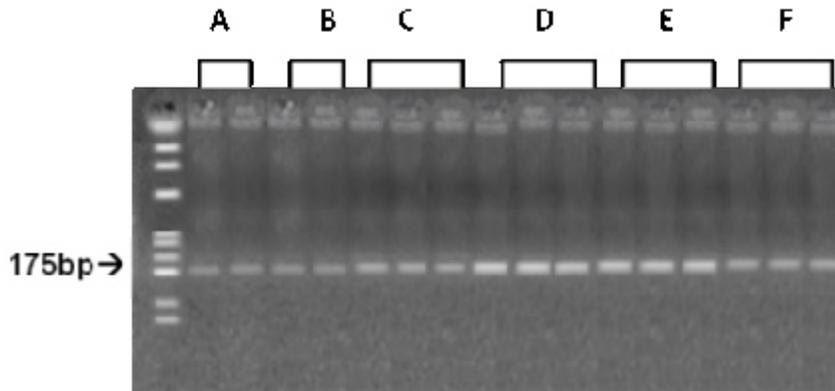


Fig. 2b: Agarose gel electrophoresis of PCR amplified Bax gene (175bp fragment) in rats treated with carcinogen+beetroots. M: DNA marker, Lanes A were loaded with DNA of the control group after 6 and 9 months, respectively. Lanes B were loaded with DNA of rats treated with beetroots for 6 and 9 months, respectively. Lanes C were loaded with DNA of rats treated with the carcinogen+ beetroots for 3, 6, 9 and 12 months, respectively

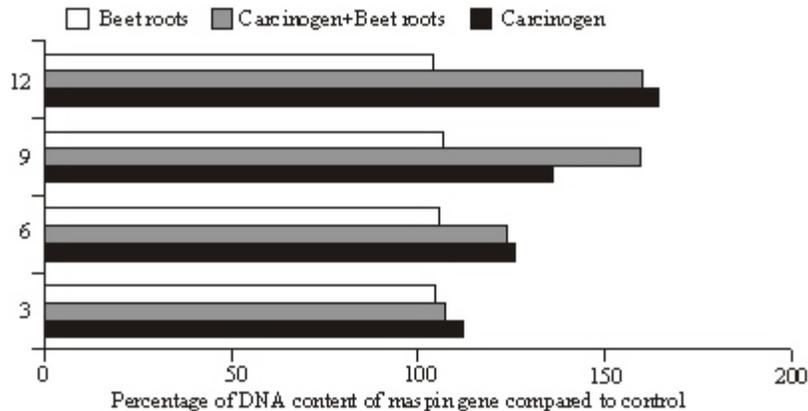


Fig. 2c: Percent of DNA content of bax gene in carcinogen, carcinogen+beetroot-treated and beet root treated rats, compared to the control levels

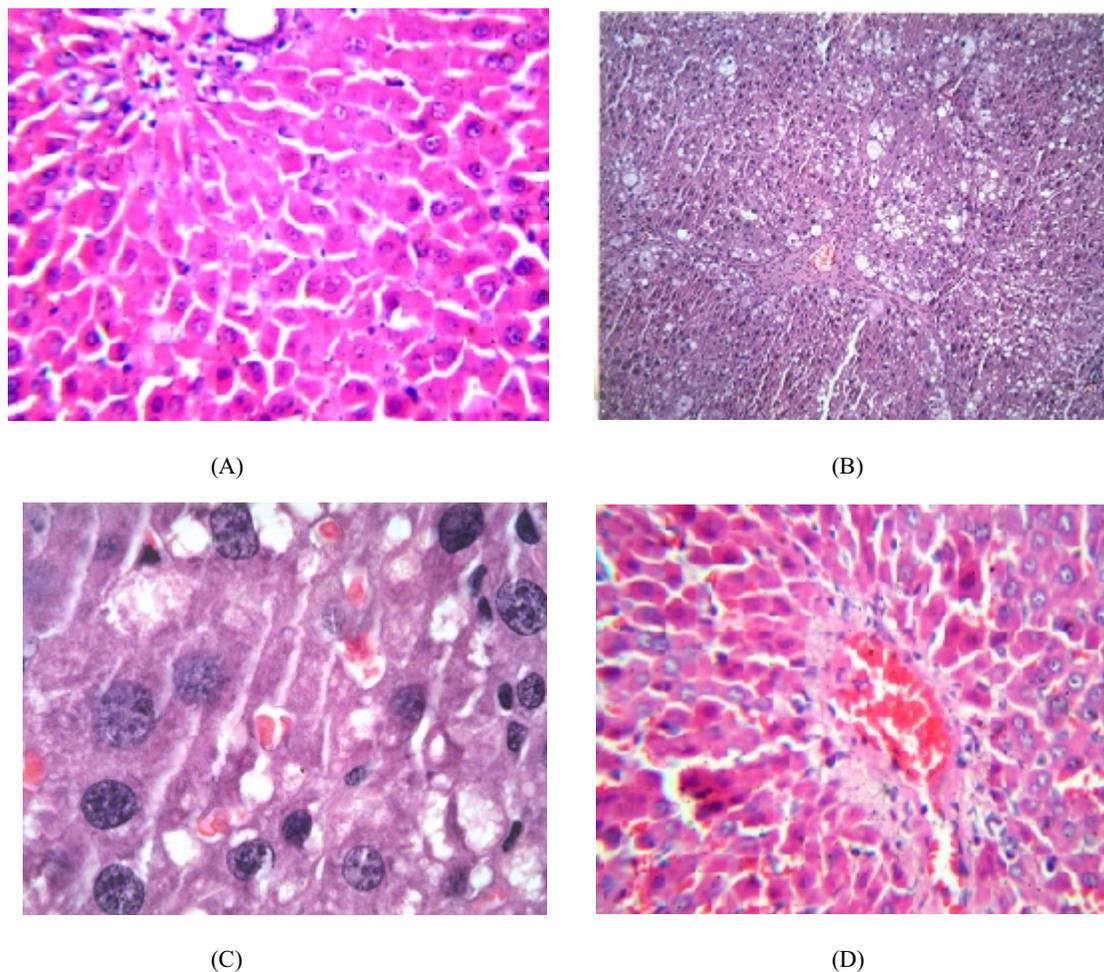


Fig. 3: Histopathological sections stained with Hx and E of liver tissues derived from rats in different groups after 12 months of treatment. (A) section of liver (40X) from normal rat (group IV) showing a normal pattern of hepatic lobule with normal blood sinusoid, (B) section of liver from rat received the carcinogen precursors in combination with (group II) showing loss of architecture, fibrosis and fatty infiltration, (C) section of liver from rat received the carcinogen precursors showing malignant nuclei

and death (Suzanne *et al.*, 2009). The oxidative stress markers investigated predicted a significant increase in the level of lipid peroxidation in rats treated with nitrosamine precursors. However, treatment with beetroots alone or in combination with nitrosamine precursors exhibited significant decrease in lipid peroxidation. The NO level has increased progressively in rats administrated with nitrosamine precursors. Beetroots intake, however have normalized the NO level. This is referred to the high nitrate content of the beet roots, is thought to be a source for the biological messenger, nitric oxide (Erman *et al.*, 2004). Increased level of lipid peroxidation was recently reported during nitrosamine precursors induced hepatocarcinogenesis (Suzanne *et al.*, 2009). In such pathological condition there is a disturbance between oxidant and antioxidant

balance, which is tilted towards oxidant side (Boitier *et al.*, 1995). The observed reduction in the level of lipid peroxidation may be referred to presence of betaine, which was found to decrease hepatic lipid peroxidation in rats (Erman *et al.*, 2004). There was a significant decrease in the level of GSH in serum of rats treated with nitrosamine precursors in agreement with the reports of Hassan and Yuosef (2010) who demonstrated that nitrosamine precursors induced hepatocellular injury was escorted by a substantial fall in GSH level.

AFP, a tumor-associated fetal protein, has long been employed as a serum fetal tumor marker to monitor disease progression (Sadik *et al.*, 2008). The significant increase observed of serum AFP was in agreement with Sadik *et al.* (2008) and Subramanian *et al.* (2007) who stated that AFP might be linked to the rat

hepatocarcinogenesis induced by nitrosamine precursors. However, rats treated with beetroots alone or in combination with nitrosamine precursors exhibited significant reduction in AFP levels compared with the nitrosamine precursors-treated group.

Maspin (mammary serine protease inhibitor) is a member of the serpin (serine protease inhibitor) family (Sager *et al.*, 1996) and has been described as a relevant factor in several types of tumors (Wang *et al.*, 2004). Previous studies have demonstrated a strong relationship between the expression of maspin and bax. The later was likely to be the key effector of maspin-mediated induction of apoptosis as indicated by the activation of cleaved caspase-3. Decreased maspin has been related to the progression of breast cancer, and the loss of its expression is regarded as a critical step during the transition from non-invasive to invasive breast cancer. Other reports have demonstrated that maspin expression was negatively associated with the progression of intrahepatic cholangiocarcinoma (IHCCA) (Romani *et al.*, 2006). A similar observation was reported by Sheng (2004), where maspin was expressed in normal human mammary epithelial cells and it was down regulated during the progression of cancer. The opposite was observed in colorectal cancer, where the gene expression was increased from colorectal non-cancerous mucosa to adenocarcinoma through adenoma (Zheng *et al.*, 2007). Herein it was observed that maspin DNA levels were negatively associated with both the precarcinogen-induced oxidative stress markers and the liver cancer marker (AFP).

The mechanism of maspin action is still controversial, where it may induce apoptosis of tumour cells. Moreover, maspin over-expression modulates tumour cell apoptosis through the regulation of Bcl-2 family proteins (Zhang *et al.*, 2005) resulting in an increased release of cytochrome C from mitochondria (Latha *et al.*, 2005).

On the other hand, bax DNA in liver tissue of rats treated with carcinogen alone and in combination with beetroots showed that normal bands of bax DNA level as compared to weak band of bax DNA in the control group. These results agree with Maurer *et al.* (1998) and Krajewska *et al.* (1996) who stated that bax expression has been observed to increase from normal mucosa to primary tumor in colorectal cancer.

In the normal rat liver, bax has been detected in hepatocytes and ductal epithelial cells (Krajewski *et al.*, 1994). Previous studies have shown that bax was over expressed from hepatocytes (Kurosawa *et al.*, 1997), whereas other reports have demonstrated that bax was expressed from ductular epithelial cells only late after Bile Duct Ligation (BDL). In man some reports have shown the down regulation of bax in HCC

(Guo *et al.*, 2002). In this work significant oxidative stress-mediated bax amplification was observed. In nitrosamine precursor-generated oxidative stress, the bax gene level was increased by about 60% compared to the normal rats. This may agree with the observation of Maurer and coworkers (Maurer *et al.*, 1998), who stated that bax expression has been observed to increase from normal mucosa to primary tumor in colorectal cancer.

Taken with the precarcinogen, beetroots were unable to compensate the decrease of maspin nor the increase of bax genes. The mechanism of maspin action is still controversial, where it may induce apoptosis of tumour cells. Moreover, maspin over-expression modulates tumour cell apoptosis through the regulation of Bcl-2 family proteins resulting in an increased release of cytochrome C from mitochondria.

Histopathological examination of the liver tissues of the different experimental groups illustrated that, nitrosamine precursors-administrated group and nitrosamine precursors in combination with beet roots-treated group showed distinct alterations after 9 and 12 months from that of beet roots-administrated group and control group, such as loss of lobular architecture, fibrosis and fatty infiltration, also the nuclei were found to be malignant, these results agree with Waer *et al.* (2005) reported that dibutyl nitrosamine serves changes in the structure of the hepatic cells of rats.

## CONCLUSION

Administration of nitrosamine precursors resulted in significant elevation of serum nitric oxide, lipid peroxidation and alpha-feto protein levels while, and it caused significant reduction in reduced glutathione levels in serum. Treatment with beetroots caused significant inhibition of serum lipid peroxidation and alpha-feto protein levels and controlled elevation of serum NO, as well as it showed significant elevation in serum GSH levels. PCR technique results showed decreased maspin DNA content in nitrosamine precursor alone and in combination with beetroots, as compared with control group. Also, increased of bax DNA content was detected in nitrosamine precursors alone and in combination with beetroots, as compared with normal group. Histopathological features investigated that treatment with nitrosamine precursors showed after 9 months and 12 months distinct alternation from that of control group. The beetroots did not show improvement to the effect of carcinogen on the liver tissues of rats.

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